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Production and regulation of different lipase activities from *Rhizopus chinensis* in submerged fermentation by lipids

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ABSTRACT

The fungal *Rhizopus chinensis* could produce several types of lipase, which were mainly intracellular. During the whole-cell lipase production by this strain in submerged fermentation, it was observed that two catalytic characteristics (hydrolytic and synthetic activity) of lipases were different with addition of lipids. The hydrolytic activity of the lipase was not induced by lipids efficaciously and could be detected regardless of whether substrate-related compounds were present. However, it was found that the induction of lipids for the synthetic activity lipase was significant, and that nearly no synthetic activity was detected while the medium contained no lipids. When only a little lipid (1 g/L) was added to medium, the synthetic activity increased sharply in the initial process of fermentation. Analysis of crude membrane-bound lipase by SDS-PAGE confirmed this induction. De novo biosynthesis of lipases, especially the lipase with synthetic activity occurred only when lipids existed. Cell growth and maltose repress the lipase production with synthetic activity, but have little influence on the lipase production with hydrolytic activity. Since the production process of mycelium-bound lipase with hydrolytic activity was different, it was reasonable to consider hydrolytic activity and synthetic activity for different application purposes. Whole-cell lipase obtained from fermentation process with high synthetic activity showed excellent catalytic ability in solvent free system on synthesis of ethylcaprylate and ethyloleate, the conversion could reach more than 90% in 5 h.

1. Introduction

In the past few years, lipases (triacylglycerohydrolases, EC 3.1.1.3) served as versatile biocatalysts capable of catalyzing numerous reactions. Among these reactions, organic synthesis reactions in non-conventional media catch more and more researchers' eyes. The main interest stems is their broad substrate specificity and exceptional high stability in organic solvents. Thus, not only hydrolysis but also synthesis reactions are possible in different media. Numerous important products, such as flavour esters [1], monoacylglycerols [2], optically pure building blocks [3] and also biodiesel [4] have been produced by these reactions. Regarded as naturally immobilized catalysts, cell-bound or intracellular microbial lipases have advantages over normal extracellular counterparts, and have attracted more attentions recently [5–12]. Despite most of studies were performed based on hydrolytic activity of these enzyme, nearly all of the mycelium-bound lipases were used in organic solvents to catalyze the synthetic reactions, due to their stability to organic solvents and thermal stability [12,13].

Hydrolytic activity and synthetic activity are often used to characterize a lipase catalytic ability, and the hydrolytic activity was mostly preferred. Generally, one lipase can catalyze its reaction in both directions, but some of enzyme can exhibit only one catalytic ability, while others show both under certain conditions. A problem, therefore occurs that synthetic activities of the enzymes in organic solvents do not correspond with the hydrolytic activities in aqueous solutions [14–17]. In order to investigate or improve the synthetic activity for lipase, studies for lipase production need to be performed based on synthetic activity. However, to our certain knowledge, scarcely has researchers investigated the lipase production in light of the synthetic activity. However, there exists also some reports about the whole-cell lipase with both hydrolytic and synthetic activity. Most of the strains were selected with high hydrolytic activity towards olive oil or tributyrin, although the ester

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synthesis ability was also studied [18,19]. However, whether the two catalytic abilities during a microorganism fermentation process are coincident is still remained unclear.

The fungal strain *Rhizopus chinensis* CCTCC M201021 was isolated from *Da Qu* (a kind of traditional leaven for production of Chinese liquor) by our laboratory, and the whole-cell lipase produced was proved to be an efficient biocatalyst in biosynthesis of short-chain fatty esters in *n*-heptane [9]. In our previous studies, we found that whole-cell lipases with hydrolytic activity and synthetic activity have different fermentation characteristics [20], and enzyme with synthetic activity in the whole-cell was located as a membrane-bound lipase by the treatments of Yatalase [21]. We also have purified two intracellular isoenzymes from this strain, the molecular weight of one enzyme is 39 kDa and another is 59 kDa. Both of them possess high hydrolytic activity, but nearly no synthetic activity was detected [22]. One of lipase gene from this strain has also been cloned and expressed in *Pichia pastoris*, the expression protein is 37 kDa, hydrolytic activity is 367 U/mL, and also no synthetic activity was detected (in press article in Chinese High Technology Letters, Chinese). From these results, it can be concluded that several types of lipases might be produced by this microorganism, the same as *Candida rugosa*, which can produce at least five types of lipase [23]. Therefore, in order to explain the difference between the whole-cell lipase with hydrolytic and synthetic activities produced by *R. chinensis* during the fermentation, and to regulate the target lipase production, the different effects of lipids on the whole-cell lipase production with two catalytic abilities (hydrolytic and synthetic) were performed in the present work. Meanwhile, the possible regulation mechanisms for the enzyme with synthetic activity were discussed.

2. Material and methods

2.1. Microorganism and culture conditions

R. chinensis CCTCC (China Center for Type Culture Collection) M201021 was studied to produce whole-cell lipase in submerged culture. The fermentation cultures were carried out in Erlenmeyer flasks (250 mL) containing 20 mL fermentation medium. The composition of basal medium contained only $MgSO_4{\cdot}7H_2O$ (0.5 g/L), K_2HPO_4 (3 g/L), maltose (0.5 g/L), peptone (40 g/L), pH was adjusted to 5.5. Different lipids were added to the basal medium with different concentration and at certain time.

After sterilization, the medium was inoculated with spores from a fresh potato dextrose agar slant (3-day-old) with spore concentration of 4.25×10^8 spores/L. Fermentations in shake flask were carried out in a rotary shaker at 30 °C under shaking speed of 200 rpm for certain time. Three flasks were taken per time for triplicate analysis. The cultivated mycelium was separated from the culture by filtration, washed twice with tap water and once with 25 mM phosphate buffer (pH 7.50), The mycelia were then lyophilized for 24 h by a freeze drying system (Labconco, USA).

2.2. Biomass determination

The biomass was determined by weighing the dry cell after freezing-dry.

2.3. Oleic acid concentration assay

Ten millilitre fermentation broth was extracted by 2 mL hexane. Free fatty acids in the organic layer were determined by colorimetric method based on cupric soaps formation [24]. The latter are soluble in hexane. Intensity of blue color was measured at 710 nm. Standard curves prepared for linoleic and oleic acid (Sigma) were used to calculated the FFA content in lipid samples.

2.4. Lipase activity assay

Synthetic activity was measured by the ester-synthesis method in heptane according to the procedure described previously [21]. Octanoic acid (1.2 M) and ethanol (1.2 M) in heptane each of 0.5 mL were mixed. The reaction was started by adding 20 mg dry cell and incubated for 30 min at 40 °C with a shaking speed of 200 rpm. The reaction mixture was then filtered using 0.15 μm membrane to remove the cells. Samples (400 μL) were then drawn and mixed with 2-hexanol of 100 μL, as internal-standard, then analyzed by subsequent gas chromatograph (Agilent 6820, flame-ionization detector, 30 m × 0.22 mm PEG 20M (AC20) capillary column, Nitrogen was used as a carrier gas, and the injector and detector temperatures were set at 250 °C. Oven temperature by programmed temperature was started at 90 °C for 1 min before being elevated to 200 °C for 5 min at 10 °C/min). One unit of lipase synthetic activity was defined as the amount of enzyme which catalyzed to produce 1 μmol of ester per minute.

Hydrolytic activity was measured by hydrolyzing olive oil. Olive oil method was modified from olive oil emulsion method described by Salleh et al. [25]. The reaction mixture comprised 2.5 mL phosphate buffer (50 mM, pH 7.5) and 2 mL olive oil emulsion (olive oil/3% polyvinyl alcohol (w/v%), 1:3 (v/v)), 10 mg mycelium-bound lipase was added to start the reaction. The reaction was carried out for 15 min at 37 °C, with shaking at 150 rpm. The reaction was terminated by adding 7.5 mL ethanol, and the amount of free fatty acid released was measured by titration to pH 10.0. A unit of activity is equivalent to one micromole of free acid released per minute.

2.5. Preparation and analysis of crude membrane-bound lipase

One gram of grounded lyophilized mycelium from different culture conditions was treated by 15 mL acetone for 15 min under room temperature. The mixture was filtrated by vacuum filtration to remove the solvents, and dried for several hours. The dry cell was washed twice with 20 mL of 2.5 mM phosphate buffer (pH 5.5) and once with 20 mL of 2.5 mM phosphate buffer contained 0.1% Trition X-100 (pH 5.5). After centrifugation at 10,000 rpm for 10 min, the precipitate was shaken in 2.5 mM phosphate buffer contained 1.5% Trition X-100 (pH 5.5) for 4 h, the volume used was 10 times of the weight of acetone-treated dry cell. Supernatant was collected as membrane-bound proteins after centrifugation at 15,000 rpm for 20 min. Sample was analyzed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 13.5% separating and 5% stacking) according to Laemmli's method [26]. Protein concentration was determined using BCA (bicinchoninic acid) [27] Protein Assay Kit (Novagen, USA).

2.6. Induction of lipases formation by washed mycelium

This experiment is modified from the method by Rapp et al. [28], mycelium for 36 h old culture of *R. chinensis* on 1% (w/v) triolein was collected, and washed with deionized water and centrifugated to remove the triolein. Then, 5 g/L washed mycelium was incubated in 50 mM potassium phosphate buffer (pH 5.5) and culture medium, 1% of triolein and oleic acid were added accordingly.

2.7. Ester synthesis in solvent free system

Ester synthesis was carried out in 10 mL screw-capped test flasks by suspending lyophilized cells (80 g/L) in solvent free system. Each flask contains equivalent molar of acid and ethanol (2.88 g caprylic